ON THE TOXICITY OF BROTH, OF PNEUMOCOCCUS BROTH CULTURE FILTRATES, AND ON THE NATURE OF THE PROTEOLYTIC ENZYME OBTAINABLE FROM PNEUMOCOCCI.*

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THE TOXICITY OF BROTHS.

Although it is well known that peptone-broth and other mixtures of protein cleavage products are toxic when injected intravenously in animals, a comparative study of the toxicity of the various kinds of broth in common use, and of the increase in toxicity of broth which results from growth of pneumococci and other bacteria in relation to the amount of amino-nitrogen, would nevertheless seem to be desirable. The source, for example, of the toxic material which is formed when bacteria are grown in broth has not been established. Is it a part of the bacterial substance, an excretion of the bacterial cell, or an action of proteolytic and other enzymes on the protein constituents of the broth itself, or a combination of various factors? In the following pages are given the results of experiments bearing on these questions.

A study of the toxicity for guinea-pigs of intravenous injection of various samples of plain broth, containing the standard amount of Witte peptone and prepared with Liebig's beef extract, shows it to be relatively slight and quite uniform. Broth prepared from meat infusion instead of beef extract not only has a greater total toxicity, but some samples are much more toxic than others. The toxicity is greater when the beef is extracted at a relatively warm temperature. The toxicity of sugar-free meat broth, which is prepared exactly as the meat broth, but rendered free from sugar by fermentation by the colon bacillus, is the most toxic of all.

An average formol titration for 10 c.c. of each of these kinds of

* Received for publication July 18, 1912.

broth expressed in N/10 KOH is 1.2 c.c. to 1.6 c.c. for the plain broth, 1.8 c.c. to 2.3 c.c. for the meat broth, and 3.2 c.c. to 3.8 c.c. for the sugar-free meat broth. It is to be remembered that the amount of Witte peptone in all three varieties is the same. The source of the increased formol titration figure and the toxicity in sugar-free broth seems to be due to the action of the colon bacillus. Thus a sample of meat infusion prepared in the usual way titrated 1.7 c.c. before and 3.6 c.c. after the colon bacillus had grown in it for 24 hours at 37° C. The toxicity was correspondingly greater. The amount of fermentable sugar in such a mixture is shown to be slight by the small increase in acidity, while the protein splitting is correspondingly greater. The toxicity, in other words, is greater when protein degradation has taken place. The symptoms produced by the injection in proper amounts of the two kinds of broth are indistinguishable from those observed following immediate anaphylaxis, or following the injection of toxic pneumococcus, of peptone, or other bacterial extracts. Those following the injection of plain broth in large amounts are not so typical, bronchial spasm being less marked, symptoms of great weakness and paralysis more marked, and death not so sudden. From these facts it is evident that there is produced during the partial digestion of the meat, the result of bacterial action, a thermostable substance which is toxic for guinea-pigs much as are pneumococcus and other bacterial autolysates.

The effect of a solution of pure amino-acids, given to me by Dr. Woelfel, was tested by intravenous injection in guinea-pigs. They showed no noteworthy immediate symptoms and remained well even though very large doses were injected. Formol titration showed the presence of approximately 10 times the amount of amino-nitrogen present in the meat broth. The biuret reaction was negative. Hence the toxic substances in broth are formed during protein cleavage, as I have shown to be the case in pneumococcus autolysates, and would seem to be intermediate protein cleavage products. It is likely, however, that other enzymes than the proteolytic are present, and it might well be that these in some way have to do with the production of toxic material.

THE TOXICITY AND PROTEOLYTIC POWER OF FILTRATES OF PNEUMOCOCCUS BROTH CULTURES.

In order to test the proteolytic power of such filtrates, toluol was added to the clear fluid of broth cultures of four strains of virulent pneumococci; the filtrates were then placed at 37° C. Titration figures of one are sufficient. The acidity remained the same throughout the experiment which shows that the splitting was not the result of growth of pneumococci. At once the titration showed 4.5 c.c.; at the end of two weeks, 6.8 c.c., and at the end of two months 7.8 c.c. No increase occurred after two months even when new, heated, ascites-broth was added. The control mixture of heated ascites-meat-broth and toluol showed no splitting. To ascertain that the protein cleavage observed in filtrates of cultures of pneumococci in broth is not due to the increased acidity, control tests were made by rendering the reaction of the filtrate the same as the broth controls by means of sodium hydrate and by bringing broth to the same acidity as the filtrates by adding hydrochloric acid. In the former, splitting took place as rapidly as when the reaction was acid, while in the latter no splitting occurred. Experiments on the relative toxicity of culture fluids of pneumococci in meat broth and the corresponding broth show that there is a regular increase in the toxicity as pneumococci grow in broth and usually a corresponding increase in amino-nitrogen. The increased toxicity is not due to the increase in acidity which is produced by growth in media containing sugar.

In a previous paper I have stated that not all the toxic substances in infectious diseases may have their origin in the bacteria themselves, but that the mere growth of bacteria in the animal juices may also produce toxic material. The question whether the toxic substance comes from the pneumococci only or whether proteolytic action on the broth by the pneumococcus or its products may also produce toxic material was then considered.

Table 1 shows that during growth of pneumococci in broth there goes into solution a proteolytic ferment which, when added to meat broth in diminishing amounts, produces a proportionate amount of protein cleavage, and at the same time a corresponding increase in toxicity up to a certain point, after which the toxicity diminishes

even though formol titration increases. A closer study of the disappearance of toxicity after protein cleavage has reached a certain point, shows that disappearance occurs regularly when the cleared culture fluid is incubated and when it is added to the meat broth in varying amounts, but does not occur when pneumococci are allowed to remain or, if at all, until a much later period. The same is true when non-virulent cultures of pneumococci are incubated for a long time. In one sample the formol titration, the toxicity, and the viability of pneumococci were tested at intervals

TABLE 1.

PROTEOLYSIS IN MEAT BROTH DUE TO BROTH CULTURE FILTRATES OF VIRULENT PNEUMOCOCCI.

MIXTURES	FORMOL TITRATION		SYMPTOMS FOLLOWING INTRAVENOUS INJECTION OF 3.5 C.C. IN (DUPLICATE) GUINEA-PIGS
	Immediately	18 Days	
40 c.c. meat broth+10 c.c. broth culture filtrate pneumococcus 622	3.0*	6.25	Definite but slight†
40 c.c. meat broth+1 c.c. broth culture filtrate pneumococcus 622	2.7	4.2	Death in four minutes
40 c.c. meat broth+10 c.c. NaCl solution	2.6	2.8	Slight

* The reaction of the mixtures showed no noteworthy change and hence is omitted in the table.

† The same quantity injected at the end of 10 days when formol titration figure was 4.8 produced death in three and four minutes. Cultures showed that the mixtures were sterile throughout the experiment.

for six months. The formol titration figure increased from 2.15 c.c. to 7.3 c.c. The toxicity remained high to the end of the experiment, and cultures showed the presence of living pneumococci in all the tests. This is interesting because filtrates of broth culture of non-virulent pneumococci contain little or no proteolytic enzyme, and it would therefore seem that here the proteolysis is due to bacterial growth.

THE PROTEOLYTIC POWER OF EXTRACTS IN NACL SOLUTION OF PNEUMOCOCCI AND OTHER BACTERIA.

I have shown previously that when extracts and suspensions of virulent pneumococci and typhoid bacilli are placed at 37° C. there is a definite increase in amino-nitrogen. This does not occur in the case of extracts of non-virulent pneumococci, of streptococci, and of staphylococci. The increase in amino-nitrogen at the end

of 48 hours in meat broth cultures of these organisms is usually greatest in the former two, and hence it appears that in these a proteolytic enzyme goes into solution.

The extracts used in the experiments in Table 2 were prepared by suspending the growth of the various bacteria from 150 c.c. meat broth in 45 c.c. NaCl solution and placing them at 37° C. for 48 hours. Ether was added at once and then allowed to evaporate through the cotton plugs. The bacteria were removed by centrifugation and filtration through Berkefeld filters. The mixtures remained perfectly clear, and cultures on blood agar remained sterile throughout the experiment. The ascites-meat-

TABLE 2.

PROTEOLYTIC POWER OF EXTRACTS IN NaCl SOLUTION OF VARIOUS BACTERIA.

MIXTURES 150 c.c. HEATED ASCITES-MEAT-BROTH TO WHICH IS ADDED 30 c.c. NaCl SOLUTION OR BACTERIAL EXTRACT AS FOLLOWS:	FORMOL TITRATION				
	Immediately	48 Hours	4 Days	7 Days	18 Days
NaCl solution	2.45	2.5	...	...	2.7
Extract of highly virulent pneumococcus (602) in NaCl solution	2.5	3.3	3.2	3.9	4.7
Extract of non-virulent pneumococcus (R51A)	2.5	2.8	2.6	...	2.65
Extract of virulent streptococcus (595)	2.7	2.8	...	2.7	...
Extract of virulent staphylococcus	2.6	2.9	3.0	...	3.0

broth was previously heated to 58° C. for 48 hours. The strain (602) of virulent pneumococcus had just been isolated from the blood of a fatal case of lobar pneumonia. The non-virulent strain (R51A) has been cultivated for 10 years. The streptococcus had been isolated from the throat in a case of scarlet fever three months previously. The staphylococcus came from a "malignant carbuncle" of the upper lip and was in the third generation. It is seen that the amino-nitrogen increased to a rather marked degree only in the broth to which was added the extract of the virulent pneumococcus. The control, the one containing extracts of the non-virulent pneumococcus and the streptococcus, remained the same while the mixture containing staphylococci showed a slight increase. The toxicity for normal guinea-pigs of the mixtures of ascites-meat-broth and NaCl solution, of the extracts of the non-virulent pneumococcus, of the streptococcus, and of the staphylococcus did not change. The mixture containing the extract of

virulent pneumococci, while equally toxic in the beginning, was twice as toxic at the end of seven days. At the end of 18 days the toxicity, while greater than in the beginning, was distinctly less than at the end of seven days. The fact that no demonstrable difference in toxicity could be made out in those mixtures which show little or no difference in titration is good evidence that no splitting took place which might not be measurable by formol titration. It appears then that extracts of virulent pneumococci contain proteolytic enzymes which not only act on the pneumococcus proteins, as is the case in the autolysate, but which have the power to split foreign proteins such as are present in ascites-meat-broth. During this process there is produced highly toxic material which in its action on guinea-pigs is the same as that obtained from pneumococci, etc.

Quantitative studies show that while the filtered extracts and broth culture filtrates increase the toxicity of meat broth and heated ascites-meat-broth, as they cause protein splitting, the increase in toxicity in proportion to the protein splitting is less than when live pneumococci are allowed to grow in the corresponding broth. The total increase in toxicity is also greater in the latter. This is what one would expect because the pneumococci furnish a share of the material from which the toxic substance is made.

THE PROTEOLYTIC ENZYME OBTAINED FROM VIRULENT PNEUMOCOCCI.

This enzyme appears to have no power to split egg white and casein, but it does increase the amino-nitrogen somewhat when added to heated human and other sera, in which it also produces toxic substances. It is interesting to note that the proteolytic power of the extracts is greatest in those fluids which are particularly good culture media for virulent pneumococci.

When filtrates of pneumococcus in broth cultures are fresh and the proteolytic enzyme is still active, heating to 60° C. for one hour and boiling markedly reduce the toxicity. Heating the broth culture suspensions does not appreciably reduce the toxicity. This confirms my former statement that when clear toxic pneumococcus autolysates are heated the toxic property rapidly disappears,

whereas autolysates containing the pneumococci may remain toxic even after boiling for 10 minutes. This is likewise true when the clear filtrates are heated after the suspensions have been kept at 37° C. for a long time and the proteolytic enzyme is no longer active. Just as the proteolytic enzyme is more resistant to heat in the fluids of broth cultures, so it is more stable on standing. Thus in three of five extracts in NaCl solution, the digestive power over ascites broth was lost at the end of five months when kept in the ice-chest, while the other two still possessed it to a slight degree, whereas broth culture filtrates, when kept under the same condition, have been found to be quite active as long as 14 months after they were made. At room temperature and in the thermostat the ferment disappears more rapidly. In this connection it should be stated that the proteolytic power of extracts of pneumonic lungs has been found to behave similarly in these respects to the extracts in salt solution.

Ether and toluol reduce the action of the proteolytic enzyme obtainable from virulent pneumococci about one-half, while chloroform-water inhibits its action almost completely but does not destroy it. Formalin and bichlorid of mercury destroy its action in 1–1,000 solutions. The optimum reaction for the action of the proteolytic enzyme is between 0.5 per cent to 1.5 per cent acid to phenolphthalein. In weak alkaline or rather strong acid solutions it is entirely inactive.

In connection with the proteolytic power and the toxicity of pneumococcus extracts a study of their effect on potato oxydase has also been made. The bluing of guaiac was used as the indicator. There exists no relation between proteolytic power and the inhibitory action on potato oxydase. Study of a long series of extracts shows that usually the inhibitory power over potato oxydase is greatest at the time when the toxicity for guinea-pigs is greatest. To this there are exceptions for which there has thus far been found no adequate explanation. A further study of the action of these extracts on oxydizing processes is contemplated.

CONCLUSIONS.

Extracts of virulent pneumococci in NaCl solution and broth culture filtrates contain a proteolytic enzyme which hydrolyzes the

proteins contained in heated ascites-meat-broth, in meat broth, and to a lesser degree those contained in heated serum. During this digestion the toxicity of the broth is increased. The action of the toxic substance obtainable in this way is identical with the action of the toxic material obtainable from pneumococci and other bacteria and with the action of peptone. It does not attack egg white or pure casein.

The enzyme is more resistant to heat and long standing in the filtrates of broth cultures than in the extracts in NaCl solution. Heating to 60° C. for one hour reduces its action approximately one-half in the former and almost completely destroys it in the latter. Ether and toluol reduce the action one-half while chloroform-water inhibits it almost completely. Formalin and bichlorid of mercury destroy it promptly.

In the mixtures freed from pneumococci the increase of toxic substance or substances is in direct proportion to the increase of amino-nitrogen up to a certain point, after which toxicity grows less although protein cleavage continues. In the mixtures, however, from which the pneumococci are not removed, the diminution in toxicity is not observed.